

Biofilm Formation, Multidrug Resistance and Clinical Risk Factors Among Uropathogenic Enterobacterales in Iraq: A Cross-Sectional Study

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Abstract:

Background: Urinary tract infections (UTIs) caused by multidrug-resistant (MDR) Enterobacterales are a growing problem in Iraq, and biofilm formation may help explain treatment failure and recurrence. Local data linking biofilm strength, resistance and clinical history are scarce. **Objectives:** To estimate the frequency of biofilm production among uropathogenic Enterobacterales, to describe antimicrobial resistance, and to test whether biofilm production and prior antibiotic use, recent hospitalization and recurrent UTI are associated with MDR. **Methods:** A prospective cross-sectional study of 200 consecutive, non-duplicate urinary isolates from symptomatic patients at Tikrit Teaching Hospital, Saladin, Iraq. Identification used standard methods; susceptibility used CLSI disk diffusion; MDR followed Magiorakos et al.; biofilm was measured by the 96-well crystal violet assay and graded as non-, weak, moderate or strong using Stepanović cut-offs. Associations were tested with chi-square, Kruskal-Wallis, trend tests and logistic regression. **Results:** Biofilm was produced by 122 isolates (61.0%): weak 17.5%, moderate 29.0%, strong 14.5%. MDR occurred in 135 isolates (67.5%), including 13 XDR (6.5%) and 4 PDR (2.0%). MDR rose with biofilm grade: 42.3% (non-producers), 68.6% (weak), 86.2% (moderate), 96.6% (strong); trend $p < 0.001$. Biofilm producers had higher MDR than non-producers (83.6% vs 42.3%; crude OR 6.95, 95% CI 3.61–13.41). In multivariable analysis, biofilm production (aOR 6.49, 95% CI 3.05–13.82), recent hospitalization (aOR 3.78, 1.57–9.10) and recurrent UTI (aOR 2.79, 1.11–7.01) were independently associated with MDR. Prior antibiotic use was independently associated with biofilm production (aOR 2.40, 1.27–4.52). **Conclusion:** Biofilm production was strongly associated with multidrug resistance among urinary Enterobacterales isolates, with MDR prevalence increasing alongside biofilm grade. Prior antibiotic use, recent hospitalization, and recurrent UTI were important associated factors.

Keywords: Urinary tract infection; biofilm; multidrug resistance; Enterobacterales.

Introduction

Urinary tract infection (UTI) is one of the most common bacterial infections, and *Escherichia coli* and other Enterobacterales cause most cases [1]. Antimicrobial resistance (AMR) compounds the problem: the Global Burden of Disease analysis estimated 4.71 million deaths associated with bacterial AMR in 2021, with carbapenem resistance rising faster than any other class among Gram-negative bacteria [2]. Forecasts suggest that by 2050 AMR could directly cause 1.91 million deaths and be associated with 8.22 million deaths a year, with the highest mortality rates expected in South Asia [3].

Iraqi studies report high resistance among urinary isolates. A 2024 retrospective review of 1,185 urine cultures from a private center found MDR in 46% of *E. coli* and 59% of *Klebsiella* isolates and described unregulated antibiotic use and the absence of a national AMR surveillance programs [4]. Hospital studies report ampicillin resistance of about 98% in *E. coli* from Al-Diwaniya [5], MDR *E. coli* and *K. pneumoniae* in women with UTI in Mosul [6], high resistance to many agents among uropathogens in Zakho [7], and carbapenemase genes, mainly *bla*OXA-48, in outpatient *E. coli* from Wasit [8].

Biofilm formation may be added to this resistance. Bacteria in a biofilm sit inside a self-produced matrix that limits antibiotic penetration, supports dormant persister cells and favors horizontal transfer of resistance genes [9–13]. Biofilm production is also linked to recurrent and catheter-associated UTI [14]. Reported biofilm prevalence in uropathogenic *E. coli* ranges widely, from 54% in Nepal [15] to 58.5% in Egyptian hospital isolates [16] and 52.85% in Baghdad [17]. Several studies have tested whether biofilm producers are more resistant [18, 19], with mixed findings, and local data that jointly consider biofilm grade, resistance category and clinical history are limited.

We therefore designed a cross-sectional study, feasible with routine laboratory equipment in an Iraqi teaching hospital, with three aims: (1) to estimate the frequency and grade of biofilm production among uropathogenic Enterobacteriaceae; (2) to describe their susceptibility profile and MDR/XDR/PDR status; and (3) to test associations between clinical factors (prior antibiotic use, recent hospitalization, recurrent UTI), biofilm production and MDR.

Materials and Methods

Design and setting

Prospective, single-center cross-sectional study at the clinical microbiology laboratory of Tikrit Teaching Hospital, Saladin, Iraq, during the period of January 20th 2025 to May 15th 2025. Reporting follows the STROBE statement [20]. The protocol requires approval by the institutional or Ministry of Health ethics committee and guardian consent, as in comparable Iraqi studies [21, 17].

Participants and isolates

We included consecutive, non-duplicate Enterobacteriaceae isolates from mid-stream or catheter urine of outpatients and inpatients with symptoms of UTI and significant growth ($\geq 10^5$ CFU/mL). Only the first isolate per patient was kept. We excluded mixed cultures with more than two organisms, asymptomatic bacteriuria, and isolates that failed to regrow.

Clinical variables

A short questionnaire and the hospital record gave age, sex, setting, diabetes, urinary catheter, and three exposures of interest:

Identification and Susceptibility testing

Isolates were identified by colony morphology on MacConkey and CLED agar, standard biochemical tests, and VITEK 2 or API 20E where available. Susceptibility to 17 agents (ampicillin, amoxicillin-clavulanate, piperacillin-tazobactam, cefotaxime, ceftazidime, cefepime, aztreonam, imipenem, meropenem, gentamicin, amikacin, ciprofloxacin, levofloxacin, trimethoprim-sulfamethoxazole, fosfomycin, tetracycline, nitrofurantoin) was tested by Kirby-Bauer disk diffusion on Mueller-Hinton agar and read with the current CLSI M100 breakpoints [22].

Biofilm assay

Biofilm was measured in 96-well flat-bottom polystyrene plates, the tissue-culture-plate/crystal violet method described by Christensen and Stepanović [24] and used in Iraqi laboratories. Overnight cultures in tryptic soy broth with 1% glucose were adjusted to 0.5 McFarland, diluted, and 200 μ L added per well in triplicate. After 24 h at 37 °C, wells were washed three times with PBS, fixed, stained with 0.1% crystal violet for 10–15 min, and the dye was solubilized in 95% ethanol. Absorbance was read at 570–620 nm on an ELISA reader. The cut-off (OD_c) was the mean of sterile-broth controls plus three standard deviations. Isolates were graded: non-producer (OD $\leq$ OD_c), weak ($\leq 2 \times$ OD_c), moderate ($\leq 4 \times$ OD_c), strong ($> 4 \times$ OD_c).

Sample size

We powered the study for the main comparison, MDR in biofilm producers versus non-producers. Assuming MDR in 80% of producers and 55% of non-producers, a producer-to-non-producer ratio of 3:1, a two-sided alpha of 0.05 and 80% powers, the standard two-proportion formula gives 102 producers and 34 non-producers (136 isolates). Adding 15% for incomplete records gives about 160. We set the target at 200 isolates (inside the 150–250 range) to allow the multivariable models and subgroup tables.

Statistical analysis

Categorical variables were compared by chi-square or Fisher's exact test; continuous variables by Welch's t-test or Mann-Whitney/Kruskal-Wallis tests. Trend in MDR across biofilm grades used the Cochran-Armitage test. Susceptibility comparisons across 17 agents were adjusted with the Holm method. Two logistic regression models were fitted, one for biofilm production and one for MDR, each with all pre-specified covariates (age, sex, inpatient status, diabetes, catheter, prior antibiotic use, recent hospitalization, recurrent UTI, *K. pneumoniae*); the MDR model also included biofilm production. Results are odds ratios with 95% confidence intervals; $p < 0.05$ was significant.

Results

The 200 isolates came from 136 women (68.0%) and 64 men; mean age was 44.2 ± 17.8 years, and 85 patients (42.5%) were inpatients. Biofilm was produced by 122 isolates (61.0%): weak in 35 (17.5%), moderate in 58 (29.0%) and strong in 29 (14.5%); 78 (39.0%) were non-producers. (Table 1).

Table 1. Characteristics by biofilm status

Characteristic	All (n = 200)	Biofilm producers (n = 122)	Non-producers (n = 78)	p-value
Age, years, mean $\pm$ SD	44.2 $\pm$ 17.8	45.5 $\pm$ 17.9	42.1 $\pm$ 17.6	0.183
Female, n (%)	136 (68.0)	82 (67.2)	54 (69.2)	0.765
Inpatient, n (%)	85 (42.5)	64 (52.5)	21 (26.9)	< 0.001
Diabetes, n (%)	43 (21.5)	34 (27.9)	9 (11.5)	0.006
Urinary catheter, n (%)	26 (13.0)	22 (18.0)	4 (5.1)	0.008
Prior antibiotic use, n (%)	111 (55.5)	80 (65.6)	31 (39.7)	< 0.001
Recent hospitalization, n (%)	63 (31.5)	39 (32.0)	24 (30.8)	0.859
Recurrent UTI, n (%)	52 (26.0)	37 (30.3)	15 (19.2)	0.081

E. coli was the most common species (58.5%), followed by *K. pneumoniae* (26.5%). Biofilm production was similar in *E. coli* (59.8%) and *K. pneumoniae* (62.3%; $p = 0.763$). MDR was 70.9% in *E. coli* and 58.5% in *K. pneumoniae* ($p = 0.110$) (Table 2).

Table 2. Species, biofilm grade and MDR, n (%)

Species	n (%)	Non	Weak	Moderate	Strong	Any biofilm	MDR
<i>E. coli</i>	117 (58.5)	47	20	35	15	70 (59.8)	83 (70.9)
<i>K. pneumoniae</i>	53 (26.5)	20	10	13	10	33 (62.3)	31 (58.5)
<i>P. mirabilis</i>	14 (7.0)	5	2	5	2	9 (64.3)	13 (92.9)
<i>Enterobacter</i> spp.	9 (4.5)	3	2	2	2	6 (66.7)	4 (44.4)

Species	n (%)	Non	Weak	Moderate	Strong	Any biofilm	MDR
<i>Citrobacter</i> spp.	4 (2.0)	1	1	2	0	3 (75.0)	3 (75.0)
Other	3 (1.5)	2	0	1	0	1 (33.3)	1 (33.3)
Enterobacterales							
Total	200	78	35	58	29	122 (61.0)	135 (67.5)

Antimicrobial susceptibility

Resistance was highest to ampicillin (73.2% of 138 tested), cefotaxime (54.0%) and ceftazidime (46.0%), and lowest to meropenem (6.0%), imipenem (7.5%) and fosfomycin (7.0%) (Table 3). Biofilm producers had higher resistance than non-producers to most agents. After Holm correction, the differences remained significant for amoxicillin-clavulanate, ceftazidime, cefepime, gentamicin, amikacin, ciprofloxacin, levofloxacin and trimethoprim-sulfamethoxazole.

Table 3. Resistance by biofilm status

Agent	Tested	Resistant, n (%)	Biofilm producers, %	Non-producers, %	P-value	Holm p
Ampicillin ¹	138	101 (73.2)	78.3	65.5	0.095	0.285
Amoxicillin-clavulanate ²	191	76 (39.8)	49.1	25.3	0.001	0.013
Piperacillin-tazobactam	200	32 (16.0)	20.5	9.0	0.030	0.242
Cefotaxime	200	108 (54.0)	61.5	42.3	0.008	0.072
Ceftazidime	200	92 (46.0)	54.9	32.1	0.002	0.019
Cefepime	200	75 (37.5)	47.5	21.8	< 0.001	0.004
Aztreonam	200	62 (31.0)	36.1	23.1	0.053	0.247
Imipenem	200	15 (7.5)	9.0	5.1	0.309	0.432
Meropenem	200	12 (6.0)	9.0	1.3	0.031	0.242
Gentamicin	200	58 (29.0)	36.9	16.7	0.002	0.023
Amikacin	200	18 (9.0)	13.9	1.3	0.002	0.023
Ciprofloxacin	200	70 (35.0)	45.1	19.2	< 0.001	0.003
Levofloxacin	200	54 (27.0)	36.1	12.8	< 0.001	0.004
Trimethoprim-sulfamethoxazole	200	75 (37.5)	47.5	21.8	< 0.001	0.004
Fosfomycin	200	14 (7.0)	9.8	2.6	0.049	0.247
Tetracycline ³	186	63 (33.9)	39.8	24.7	0.033	0.242
Nitrofurantoin ³	186	25 (13.4)	15.9	9.6	0.216	0.432

¹ Excludes intrinsically resistant *Klebsiella* and *Enterobacter* (biofilm 83 vs non-producers 55 tested). ² Excludes *Enterobacter* (116 vs 75). ³ Excludes *P. mirabilis* (113 vs 73). Holm correction across 17 comparisons.

MDR, XDR and PDR

MDR (including XDR and PDR) was found in 135 isolates (67.5%): 118 MDR only, 13 XDR (6.5%) and 4 PDR (2.0%). MDR increased steadily with biofilm grade (Table 4; chi-square 42.99, 3 df, $p < 0.001$; trend $z = 6.46$, $p < 0.001$). Biofilm producers had MDR in 102 of 122 isolates (83.6%) versus 33 of 78 non-producers (42.3%; crude OR 6.95, 95% CI 3.61–13.41). XDR or PDR were found in 15

producers (12.3%) and 2 non-producers (2.6%; $p = 0.016$). Median MAR index was 0.29 (IQR 0.19–0.47) in producers and 0.18 (0.06–0.25) in non-producers ($p < 0.001$).

Table 4. Resistance burden by biofilm grade

Biofilm grade	n	MDR, (%)	n XDR or PDR, n (%)	Median resistant categories	MAR median (IQR)	index,
Non-producer	78	33 (42.3)	2 (2.6)	2	0.18 (0.06–0.25)	
Weak	35	24 (68.6)	4 (11.4)	3	0.24 (0.15–0.41)	
Moderate	58	50 (86.2)	6 (10.3)	4	0.29 (0.24–0.41)	
Strong	29	28 (96.6)	5 (17.2)	5	0.38 (0.29–0.56)	

MAR index differed across grades (Kruskal-Wallis $H = 40.78$, $p < 0.001$).

In crude analysis, MDR was more frequent after prior antibiotic use (74.8% vs 58.4%; $p = 0.014$), recent hospitalization (82.5% vs 60.6%; $p = 0.002$) and in recurrent UTI (82.7% vs 62.2%; $p = 0.007$). Biofilm production was more frequent after prior antibiotic use (72.1% vs 47.2%) and in recurrent UTI (71.2% vs 57.4%), but not after hospitalization (61.9% vs 60.6%).

In multivariable analysis, prior antibiotic use was the only independent predictor of biofilm production (Table 5). For MDR, biofilm production, recent hospitalization and recurrent UTI were independent predictors; prior antibiotic use lost significance once biofilm was in the model (Table 6). Each one-step rise in biofilm grade (none, weak, moderate, strong) raised the odds of MDR by a factor of 2.98 (95% CI 1.98–4.48; $p < 0.001$) after adjustment.

Table 5. Factors associated with biofilm production (122 producers, 78 non-producers)

Factor	Crude OR (95% CI)	p-value	Adjusted OR (95% CI)	p-value
Age, per 10 years	1.12 (0.95–1.31)	0.184	1.06 (0.88–1.27)	0.572
Female sex	0.91 (0.49–1.68)	0.765	0.89 (0.45–1.74)	0.724
Inpatient	3.00 (1.62–5.53)	< 0.001	1.97 (0.96–4.04)	0.063
Diabetes	2.96 (1.33–6.59)	0.008	2.35 (0.99–5.60)	0.053
Urinary catheter	4.07 (1.35–12.31)	0.013	2.77 (0.83–9.28)	0.098
Prior antibiotic use	2.89 (1.60–5.20)	< 0.001	2.40 (1.27–4.52)	0.007
Recent hospitalization	1.06 (0.57–1.95)	0.859	0.79 (0.39–1.60)	0.516
Recurrent UTI	1.83 (0.92–3.62)	0.083	1.65 (0.78–3.48)	0.187
<i>K. pneumoniae</i>	1.08 (0.56–2.05)	0.826	1.05 (0.51–2.15)	0.891

Model discrimination (AUC) 0.74.

Table 6. Factors associated with MDR (135 MDR, 65 non-MDR)

Factor	Crude OR (95% CI)	p-value	Adjusted OR (95% CI)	p-value
Age, per 10 years	1.07 (0.90–1.26)	0.430	1.02 (0.83–1.26)	0.819
Female sex	0.83 (0.43–1.57)	0.560	0.97 (0.45–2.09)	0.933
Inpatient	2.87 (1.50–5.48)	0.001	1.29 (0.56–2.98)	0.545
Diabetes	2.09 (0.94–4.68)	0.071	1.34 (0.50–3.56)	0.558
Urinary catheter	4.24 (1.23–14.70)	0.023	1.89 (0.43–8.29)	0.400
Prior antibiotic use	2.11 (1.16–3.85)	0.015	1.37 (0.65–2.86)	0.410
Recent hospitalization	3.08 (1.47–6.42)	0.003	3.78 (1.57–9.10)	0.003
Recurrent UTI	2.91 (1.32–6.42)	0.008	2.79 (1.11–7.01)	0.029

Factor	Crude OR (95% CI)	p-value	Adjusted OR (95% CI)	p-value
<i>K. pneumoniae</i>	0.58 (0.30–1.12)	0.104	0.48 (0.22–1.05)	0.067
Biofilm producer	6.95 (3.61–13.41)	< 0.001	6.49 (3.05–13.82)	< 0.001

Model discrimination (AUC) 0.82.

Discussion

In this study, biofilm production was common (61.0%) and tracked closely with MDR, which rose from 42.3% in non-producers to 96.6% in strong producers. The 61.0% prevalence of biofilm sits within the range we found and it is agreeable with a previous Iraqi studies report 55% in *E. coli* on (Congo red agar) [23], 52.85% in Baghdad [17], and moderate producers in 69.2% of Baghdad isolates [21]. Outside Iraq, 54.1% was reported in Nepal [15] and 58.5% in Egyptian nosocomial isolates [16]. Differences in media, incubation and cut-offs make direct comparison difficult, which is why the protocol fixes the Stepanović method [24].

The strong association with MDR agrees with Baghdad study in which all UPEC produced biofilm at weak to moderate levels [17]. Other studies have examined the same link between biofilm and resistance [18, 19]. The evidence is not uniform: the Egyptian hospital study found only gentamicin resistance independently linked to biofilm [16], and a Nepalese study found a weak correlation between biofilm and ESBL production ($r = 0.157$) [15].

Prior antibiotic use was the clearest correlate of biofilm in our data (OR 2.40), echoing the Egyptian finding that prior therapy and diabetes were the main risk factors for biofilm-producing UPEC [16]. Recent hospitalization and recurrent UTI were the independent correlates of MDR. This is consistent with an Iraqi study identifying a UTI in the previous six months and recurrent UTI as risk factors for ESBL-producing isolates [25], and with Saudi pediatric data in which antibiotic use in the previous three months was associated with ESBL-producing *E. coli* UTI [26]. Prior antibiotic use lost significance once biofilm was in the MDR model. One caution is that Whelan and colleagues found biofilm capability could not be predicted from clinical details alone [27], so the laboratory assay, not history, is the way to detect it. *K. pneumoniae* showed lower MDR than *E. coli* (58.5% vs 70.9%).

Conclusions:

Biofilm production was common among urinary Enterobacterales isolates and was strongly associated with multidrug resistance, with MDR prevalence increasing alongside biofilm grade. Prior antibiotic use was independently associated with biofilm formation, while recent hospitalization and recurrent UTI were independent predictors of MDR. These findings highlight the importance of routine antimicrobial susceptibility testing and biofilm assessment in selected cases to support effective UTI management.

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